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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(54) Title: ASSAY FOR DETECTING AN ANALYTE CONTAINING OR LABELLED WITH A HAEM MOIETY

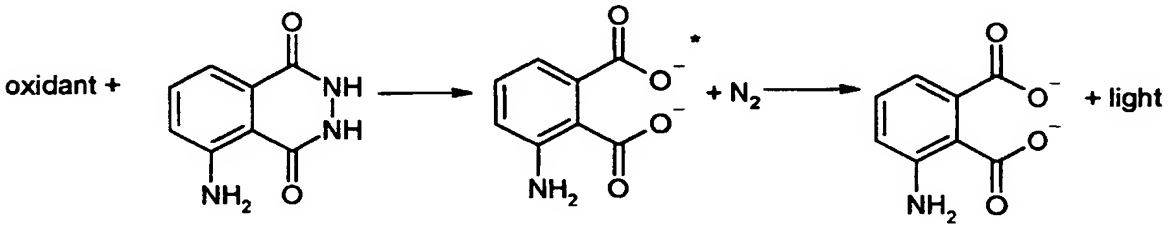
(57) Abstract: A method for detecting an analyte containing or labelled with a haem moiety within a sample, said method comprising: a) contacting said sample with a magnetic bead having immobilised thereon a specific binding partner for said analyte and allowing analyte to bind to said specific binding partner; b) separating the magnetic beads from the sample, and if necessary, labelling the immobilised analyte with a haem containing label; c) resuspending the beads and subjecting them to alkaline conditions sufficient to release haem moieties therefrom but not to extract inorganic iron from the beads; d) detecting released haem moieties using a luminol chemiluminescent assay procedure.

ASSAY FOR DETECTING AN ANALYTE CONTAINING OR LABELLED WITH A HAEM MOIETY

The present invention relates to an assay method, in particular an assay for detecting an analyte containing a haem moiety within a sample.

The haem group is a prosthetic group associated with certain cellular proteins. It is built around an atom of iron, and it is conveniently detected using a light emitting luminol chemiluminescent reaction.

In this reaction, luminol (3-aminophthalazide), or a functional chemiluminescent derivative thereof, is oxidised by an oxidant in a basic aqueous solution to generate a light emitting species (3-aminophathalate) as illustrated.



The reaction is catalysed by metal cations, in particular, the "organic" iron, present in haem molecules, to increase light emission or to increase the speed of oxidation of luminol to the light emitting species and therefore the onset or intensity of light production.

It may be used therefore in assays for the haem molecule. Functional chemiluminescent derivatives of luminol are known in the art.

However, the reaction may be highly sensitive to a variety of contaminants, and therefore separation of analyte from the source of possible contaminants is highly desirable. In particular, inorganic iron can also initiate this light emitting reaction. It has been reported for example, that Fe(III) interferes

positively with the reaction at some concentrations and negatively at others, whilst Fe(II) shows significant positive interference (Yuan J et al., Anal. Chem. 1999, 71, 1975-1980).

Consequently, it is generally recognised that the inorganic iron contaminants are particularly undesirable in a solution that is being tested in this way and could lead to false positive or negative results.

Magnetic bead separation is a particularly useful way of concentrating analytes within a sample. Specific binding partners for a particular analyte may be immobilised on the ferromagnetic beads, which are then contacted with a sample suspected of containing the analyte. Analyte becomes bound to the beads, which may then be separated from the bulk sample using magnetic separation methods, to attract the ferromagnetic beads. Once separated, analyte can be released from the beads in a more concentrated form, and detected.

However the conditions required to release a haem moiety from the analyte-bead complex is generally chemically quite stringent, and it is expected that these would be accompanied by extraction of at least some inorganic iron from the beads.

Such separation methods therefore are contraindicated for use in the luminol reaction, since the contamination risk from inorganic iron is significantly higher.

The applicants have found however, that this combination can be successfully carried out.

According to the present invention there is provided a method for detecting an analyte containing or labelled with a haem moiety within a sample, said method comprising:

a) contacting said sample with a magnetic bead having immobilised thereon a specific binding partner for said analyte and allowing analyte to bind to said specific binding partner;
b) separating the magnetic beads from the sample, and if necessary, labelling the immobilised analyte with a haem containing label;
c) resuspending the beads and subjecting them to alkaline conditions sufficient to release haem moieties therefrom but not to extract inorganic iron from the beads;
d) detecting released haem moieties using a luminol chemiluminescent assay procedure.

In particular, step (c) is conducted within a pH range of from 12.5-13.5. This is suitably achieved using a buffer or a working solution which is preferably the working solution of alkaline luminol. This solution suitably combines NaOH that causes the release of the haem moiety and luminol or an equivalent functional chemiluminescent reagent in a single solution.

The applicants have found that step (d) may be carried out directly on the bead suspension. The nature of the light emitted is so strong, that the presence of the beads does not detract from the signalling process. However, if desired, after step (c), the magnetic beads can be separated, and step (d) is carried out on supernatant remaining.

Suitably the beads are subjected to one or more washing steps between step (b) and step (c). In these, the magnetic beads are resuspended in a washing solution, and thereafter, separated from the washing solution.

Resuspension of beads during these washing steps, as well as during step (c) may be carried out using conventional methods, such as by using a whirlimixer, but is preferably carried out using relatively gentle methods such as pipetting, in order to minimise loss of bound material from the beads.

The method of the invention is particularly suitable for the detection of analytes such as spores, in particular *Bacillus globigii* (BG) spores, which may be more difficult to detect using other assay methods, because of the relative difficulty of accessing cellular materials, in particular proteins such as enzymes.

Alternatively, where the target analyte does not contain a haem moiety, it may be labelled with a haem containing second specific binding partner such as an antibody. Some enzymatic labels commonly used in immunoassays, such as horseradish peroxidase (HRP) contain a haem prosthetic group. Using the method of the invention, a non-haem containing analyte may be a protein or peptide, which is bound to an antibody-coated bead. After initial capture, an HRP labelled antibody is added to the separated beads after step (b) to introduce a haem label onto any immobilised analyte. It is then necessary to separate and resuspend the beads, optionally with a washing step between steps (b) and (c). In this embodiment, the haem moiety is extracted from the antibody which forms a "sandwich" with any analyte immobilised on the beads.

Suitably, in step (d), luminol is first added to the released haem moieties in the alkaline conditions of the buffer, suitably in excess, and incubated with them, and thereafter, oxidant added in a sufficient quantity to generate a signal. Suitable oxidants include those known in the art, including peroxides, such as hydrogen peroxide, perborate, permanganate or hypochlorite salts, for example of alkali metals such as sodium or potassium, or iodine. Preferably the oxidant used is a perborate, and in particular, sodium perborate. Another preferred oxidant is hydrogen peroxide.

The oxidant and the luminol, or functional chemiluminescent derivative thereof, are suitably added in a significant amount compared to the likely concentration of haem in the solution.

This means that the signal generated can be related to the amount of haem present in the sample acting as a catalyst for the reaction, and the magnitude of the signal is not limited by the lack of luminol or oxidant.

For instance, where for example a 0.005%w/w luminol solution is used in the reaction, the ratio of the volume of the luminol solution added to the reaction: the volume of reagent solution is in the range of from 1:2 to 10:1 and preferably about 1:1. Where the luminol solution used is of a different concentration, the volume ratio will be varied accordingly to provide equivalent relative amounts of the reactants.

The amount of oxidant added will depend upon the particular oxidant used, but it should suitably be sufficient to oxidise all of the luminol present in the reaction.

Immobilisation of specific binding partners such as antibodies or binding fragments thereof, onto the magnetic beads may be carried out using any of the conventional procedures. Step (a) is suitably carried out by incubating the coated beads with a solution of the sample for a sufficient period of time, and at a suitable temperature, for example at about 37°C, to allow good capture of the analyte by the specific binding partner.

Suitably, the concentration of magnetic beads used is sufficient to ensure good capture efficiency of the analyte. Thus the concentration of beads used is suitably in the range of from about $1x10^4$ bead.ml^{-1} to $1x10^8$ beads.ml^{-1}.

Similarly, the amount of liquid added during the resuspension in step (c) is kept low in order to provide improved concentration factors.

The method of the invention has been found to have very good sensitivity, in particular for spores as analytes. Furthermore,

the strength of the signal obtainable in this way means that no amplification step is required, so it provides a very rapid assay. In addition, it may be detected by a wide range of detectors, including photodiodes.

Reagent costs for this assay are very low compared to say bioluminescent assay systems.

Furthermore, by coupling the assay to the IMS capture, the inherent susceptibility of the chemiluminescent assay to interferents is reduced.

The invention further provides a kit for carrying out the method of the invention. In particular the kit will comprise magnetic beads, luminol, or functional chemiluminescent derivatives thereof and a working solution having a pH within the range of from 12.5-13.5.

The working solution is preferably the working solution of alkaline luminol and may be a buffer. This solution suitably combines the NaOH required for the release of the haem moiety and the luminol or functional chemiluminescent reagent in a single solution.

Suitably, the magnetic beads are coated with a specific binding partner for an analyte. Particular examples of specific binding partners include antibodies or binding fragments thereof.
In addition, the kit may further comprise an oxidant for luminol as described above, and in particular, sodium perborate.

As described hereinafter, Dynal tosylated beads were coated with CBD rabbit anti-BG antibody and used to capture *Bacillus globigii* spores. A chemiluminescent assay end point was successfully used as an alternative to those that produce a bioluminescent signal.

Surprisingly, initial tests on "naked" beads indicated that uncoated beads did not give significant blank readings with the chemiluminescent assay. Later tests with antibody coated beads did produce higher readings than the blank measurements (resuspension buffer). It was found that the background signals were not prohibitively high, even when doing the assay in a PMT based luminometer, which is generally regarded as being oversensitive for chemiluminescence work.

The invention will now be particularly described by way of example.

Example

Method and materials

Instrumentation

All luminometric measurements were made in a TL Plus Luminometer from Thermo Life Sciences (Basingstoke, UK), using 3.5mL polystyrene tubes obtained from Biotrace (Bridgend, UK). 1-second delay interval and 1 second measurement duration was used in all measurements.

Reagents

Luminol was obtained from Fluka (Poole, UK), EDTA and sodium perborate was obtained from BDH (Poole, UK), sodium hydroxide, phosphate buffer, tris buffer, PBS Tween buffer were obtained from Sigma (Poole, UK). Sterile distilled water and sterile phosphate buffered saline were obtained from Gibco (Paisley, UK). Tryptone soya agar plates were obtained from Oxoid (Basingstoke, UK). 280µm tosyl and epoxy activated paramagnetic beads were obtained from Dynal (UK) Ltd (Wirrall, UK). Rabbit anti-BG polyclonal antibodies and rabbit anti-*E coli* polyclonal antibodies were obtained from DSTL Detection & Diagnostics antibody group.

A stock solution of alkaline luminol (100g sodium hydroxide, 37.5g EDTA, 5g luminol dissolved in 1 litre of sterile distilled

water) was prepared and kept in a (light proof) container at 4°C. Working solution of alkaline luminol was prepared by diluting 8ml of the above to 100ml with water. Sodium perborate solution was prepared by dissolving 1g of sodium perborate and 0.1g EDTA in 100ml of sterile distilled water.

Paramagnetic beads were coated using the manufacturers' coating procedures. Antibody conjugation to the tosyl-activated beads was performed in Buffer A (100mM sodium phosphate buffer, pH 7.4). Conjugation was performed for 24 hours at 37°C using a Dynal mixing wheel.

Immunomagnetic separation

A 10-fold dilution series in PBS was prepared from stock BG spore suspension at a concentration of 1.0E+11 cfu.ml^{-1}. 1000µl of the test dilution was dispensed into sterile Eppendorf tubes. Anti-BG coated magnetic beads were added to each sample to give a final concentration of ~1.0E+07 beads.ml^{-1}. Anti-*E coli* coated magnetic beads were added to duplicate samples as a control. The samples were incubated at 37°C for 10 minutes on a Dynal mixing wheel (18 rpm).

On removal from the mixing wheel the samples were placed into a Dynal Magnetic Particle Concentrator (MPC) and the magnet was applied. After 2 minutes the unbound supernatant was removed (and retained for plate count assays) after which the magnet was removed from the rack.

The beads were then resuspended by the gentle addition of 1000µl of PBS or PBS containing 0.05 % Tween 20. The magnet was applied to the rack and after 2 minutes the unbound supernatant was removed and discarded.

The magnet was then removed from the rack and the beads were resuspended in 1000µl PBS, although the final resuspension volume was varied depending on the final assay requirements.

Assay

100µl aliquots of the bead suspensions resulting from the immunomagnetic separation protocol were removed and placed into 3.5mL polystyrene tubes. 100µl of the working luminol solution were added to the sample and were incubated (at room temperature) for 1 minute. 100µl of sodium perborate solution were added and the light generated by the reaction was immediately measured in the luminometer.

Plate counts

Bacterial CFU counts on the samples, were performed by plating out 100µL of the sample on to tryptone soya agar plates in triplicate and incubating these for 24 hours at 37°C before counting.

Using the combination of an immuno-magnetic capture and separation with a chemiluminescent endpoint assay, a specific detection limit of 6.0E+06 cfu/ml unwashed BG spore was demonstrated.

Claims

1. A method for detecting an analyte containing or labelled with a haem moiety within a sample, said method comprising:
a) contacting said sample with a magnetic bead having immobilised thereon a specific binding partner for said analyte and allowing analyte to bind to said specific binding partner;
b) separating the magnetic beads from the sample, and if necessary, labelling the immobilised analyte with a haem containing label;
c) resuspending the beads and subjecting them to alkaline conditions sufficient to release haem moieties therefrom but not to extract inorganic iron from the beads;
d) detecting released haem moieties using a luminol chemiluminescent assay procedure.

2. A method according to claim 1 wherein in step (c) is conducted within a pH range of from 12.5-13.5.

3. A method according to claim 1 or claim 2 wherein step (d) is carried out directly on the bead suspension.

4. A method according to claim 1 or claim 2 wherein after step (c), the magnetic beads are separated, and step (d) is carried out on supernatant remaining.

5. A method according to any one of the preceding claims wherein between step (b) and step (c), the magnetic beads are resuspended in a washing solution, and thereafter, separated from the washing solution.

6. A method according to any one of the preceding claims wherein the analyte is a spore.

7. A method according to claim 6 wherein the analyte is a *Bacillus* spore.

8. A method according to any one of claims 1 to 5 wherein the analyte is labelled with a haem containing moiety.

9. A method according to claim 8 wherein said haem containing moiety is a horseradish peroxidase labelled antibody specific for an analyte.

10. A method according to any one of the preceding claims wherein in step (d) luminol is added to the released heam moieties and incubated therewith, and thereafter, oxidant added to generate the signal.

11. A method according to claim 10, wherein amount of oxidant present is sufficient to oxidise all of the luminol.

12. A method according to claim 10 or 11 wherein the oxidant is sodium perborate or hydrogen peroxide.

13. A method according to any one of the preceding claims wherein the specific binding partner for the analyte is an antibody or binding fragment thereof.

14. A kit for use in a method according to claim 1, said kit comprising magnetic beads, luminol or functional chemiluminescent derivatives thereof and a working solution having a pH within the range of from 12.5-13.5.

15. A kit according to claim 14 wherein said magnetic beads are coated with a specific binding partner for an analyte.

16. A kit according to claim 15 wherein said specific binding partner is an antibody.

17. A kit according to any one of claims 14 to 16, which further comprises an oxidant for luminol.

18. A kit according to claim 17 wherein the oxidant is sodium perborate or hydrogen peroxide.

19. A method according to claim 1 substantially as hereinbefore described with reference to the Example.

INTERNATIONAL SEARCH REPORT

Inter ıal Application No
PCT/GB 03/02716

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/543 G01N33/58

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MÜLLER-SCHULTE D ET AL: "Novel magnetic microspheres on the basis of poly(vinyl alcohol) as affinity medium for quantitative detection of glycated haemoglobin." JOURNAL OF CHROMATOGRAPHY. A. NETHERLANDS 8 SEP 1995, vol. 711, no. 1, 8 September 1995 (1995-09-08), pages 53-60, XP002253221 the whole document	1-18
Y	WO 98 54578 A (HIXSON CRAIG S ;BIO RAD LABORATORIES (US)) 3 December 1998 (1998-12-03) example 2	1-18

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[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 September 2003	30/09/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Diez Schlereth, D

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int[illegible]al Application No
PCT/GB 03/02716

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	PATENT ABSTRACTS OF JAPAN vol. 1995, no. 09, 31 October 1995 (1995-10-31) & JP 07 140143 A (INAX CORP), 2 June 1995 (1995-06-02) abstract	1-18
Y	EP 0 480 361 A (TAKEDA CHEMICAL INDUSTRIES LTD) 15 April 1992 (1992-04-15) page 5, line 12 -page 6, line 30	1-18
A	US 5 156 971 A (ALLS JOHN L ET AL) 20 October 1992 (1992-10-20) abstract	1-18
A	EWETZ L ET AL: "FACTORS AFFECTING THE SPECIFICITY OF THE LUMINOL REACTION WITH HEMATIN COMPOUNDS" ANALYTICAL BIOCHEMISTRY, ORLANDO, FL, US, vol. 71, no. 2, 1976, pages 564-570, XP000618259 ISSN: 0003-2697 the whole document	1-18

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte inal Application No
PCT/GB 03/02716

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9854578 A	03-12-1998	AU 7487798 A	30-12-1998
		WO 9854578 A1	03-12-1998
JP 07140143 A	02-06-1995	NONE	
EP 0480361 A	15-04-1992	CA 2053120 A1	12-04-1992
		EP 0480361 A2	15-04-1992
		JP 5001995 A	08-01-1993
US 5156971 A	20-10-1992	NONE	

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 03 02716

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 19

The wording "substantially as herein described..." together with the lack of any distinguishing technical feature renders the scope of claim 19 so unclear (Art. 6 PCT) that it is not possible to carry out a meaningful search encompassing the whole scope of this claim.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

ational application No.
PCT/GB 03/02716

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 19
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.